J. Cell Commun. Signal. (2017) 11:147–153
DOI 10.1007/s12079-016-0366-2

RESEARCH ARTICLE

Crosstalk between SHH and stemness state signaling pathways in esophageal squamous cell carcinoma

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Received: 1 September 2016 / Accepted: 22 November 2016 / Published online: 30 November 2016

Abstract The expression of GLI1 as a downstream gene of sonic hedgehog (Hh) pathway, studied in a variety of cancers including esophageal squamous cell carcinoma (ESCC). However, the interaction of Hh with other developmental pathways needs to be elucidated. In this study, we aimed to investigate the correlation of GLI1 expression with transcription factors (TFs) of stem cell signaling pathways, and their association with clinico-pathological data of ESCC. Using real-time PCR, we assessed the expression of GLI1 mRNA in 49 ESCC patients, and analyzed the correlation between GLI1 and selected TFs. The results showed overexpression of GLI1 in ESCC tissues in significant correlation with lymph node metastasis. The GLI1 up-regulation was also correlated to the SOX2 and SIZN1 (Smad-interacting zinc finger protein) expression. These correlations may confirmed the role of GLI1 in crosstalk among different cell signaling pathways in ESCC. To our knowledge, this is the first study to demonstrate the correlation of GLI1 expression with stemness marker and BMP signaling in ESCC.

Keywords GLI1 overexpression · Stemness · SIZN1 · SOX2 · ESCC

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Introduction

Esophageal cancer (EC) is the sixth cause of mortality in the world. The most prevalence type of EC in Asian countries is esophageal squamous cell carcinoma (ESCC) (Zhang 2013). Since the diagnosis of the disease is usually performed at advanced stages of the malignancy, the 5-year survival rate after surgery is approximately 35%. Therefore, it seems necessary to recruit the molecular mechanisms of cancer progression in prevention, diagnosis, and therapeutic interventions of cancers (Forghanifard et al. 2014a). The molecular investigation are carrying out in different aspects of cellular mechanisms to help understanding the system biology of ESCC (Dadkhah et al. 2013; Moghbeli et al. 2016a, b).

Hedgehog (Hh) signaling is normally involved in development, differentiation of embryonic tissues, and hemostasis of normal adult cells, as well as carcinogenesis (Rimkus et al. 2016). Canonical Hh signaling initiates by binding of the Hh ligands, most likely by Sonic HH (SHH), to its receptor called patched (Ptch). Smoothened (Smo), a potential G protein-coupled receptor (GPCR), is subsequently alleviated from Ptch, which mediates Smo repression. Smo signal transduction finally leads to increased expression and activation of Glioma-associated oncogene homolog 1 (Gli-1) (Ingham 2008). Mutations in different transcription factor of Hh signaling as well as deregulation of its target genes give rise to a variety of disorders such as Gorline syndrome and basal cell carcinoma (Evangelista et al. 2006). On the other hand, growing evidences

elucidated the non-canonical regulation of GLI1, SMO-independent, which is modulated by diverse signaling cascades including TGF ß, NOTCH, SMAD and RAS (Aberger et al. 2012).

GLI1, as a downstream gene of Hh canonical signaling, culminate to control the TFs of Hh pathway target genes. It activates Cyclin D2 and FOXM1 in basal cell carcinoma, and also has oncogenic role in different malignancies including ESCC (Yoshikawa et al. 2008; Wei and Xu 2011; Min et al. 2013; Mori et al. 2007), papillary thyroid (Bian et al. 2014; Lee et al. 2015), hepatocellular, and head and neck squamous cell carcinomas (Dimitrova et al. 2013; Gai et al. 2014), as well as breast (Xu et al. 2010), ovarian (Ke et al. 2015), and cervical cancers (Nayak et al. 2016). The correlation of GLI1 with PI3K/AKT – MAPK and WNT signaling pathways, has role in proliferation of ESCC and colon cancer, respectively (Wei and Xu 2011; Varnat et al. 2010) . SHH is correlated to the NOTCH pathway in medulloblastoma cancer stem cell (CSC) populations through regulation of GLI1 expression with HES1 (Cordeiro et al. 2014; Schreck et al. 2010). The overexpression of GLI1 was linked to lymph node metastasis and EMT of ESCC patients (Min et al. 2013; Mori et al. 2007). Nevertheless, the correlation of GLI1 expression with stemness state of ESCC and EMT induction is not clear yet. Therefore, we aimed to analyze the expression of GLI1 in ESCC patients and elucidate its correlation with TFs of stemness state, as well as their association with demographic data of ESCC patients.

Material and methods

The 49 ESCC with related margin normal tissues were collected form patients who referred to Oncology Omid Hospital, Mashhad, Iran. All patients did not receive preoperative chemo- and radiotherapy experiences before surgery. The pathological features were categorized based on the seventh edition of Union International Cancer TNM classification guidelines (Sobin et al. 2011). The study were approved by the MUMS ethics committee and all patients declared their informed consent.

RNA isolation and qRT-PCR

RNA was extracted from tissues using TriPure RNA extraction reagent (Roche, Nutley, NJ). The cDNA synthesis was performed by PrimeScript First Strand cDNA Synthesis Kit (Takara, japan). The cDNA was amplified by SYBR green method and specific primer sets (Table 1) in Stratagene Mx-3000P real-time thermocycler (Stratagene, La Jolla, CA) to compare gene expression. ROX was used as reference dye. The following optimal thermal condition was used: 10 min at 95 0C, 39 cycles of 15 s at 95 0C, 20 s at 56 0C, and 45 s at 72 0C. All experiments were performed in triplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as normalizer. The PCR efficiency for the genes was measured using standard curves and the equation $E = 2^{(-\Delta\Delta CT)}$ was used to measure fold change of genes expression (Raeisossadati et al. 2011; Khales et al. 2015). Based on this equation, more than 2 fold change of mRNA expression in tumors in comparison with related normal tissues was introduced as overexpression, while less than −2 fold change was considered as underexpression. The range between 2 and −2 was described as no change or normal expression.

Statistical analysis

Using SPSS 23 statistical package (SPSS, Chicago, IL), the data were analyzed. The $\chi 2$ or Fisher exact test, independent-sample t test and ANOVA were used to evaluate the correlation of gene expression with clinicopathological data. The Pearson's test was used to correlate between GLI1 and other genes expression. $P < 0.05$ was considered statistically significant.

Results

Study population

23 males and 26 females were recruited in this study. The mean age ± standard deviation (SD) of patients was 61.40 ± 12.10. The range of tumor size in samples was 1.50 to 12.00 (Mean ± SD: 4.17 ± 1.90) which resected from lower, middle or upper parts of esophagus. The tumoral characteristics of tissues were histologically confirmed by pathologist. The clinicopathological features of the patients are presented in Table 2.

Table 1 Primer sequences used for qRT-PCR

Gene	Forward Primer	Reverse primer	Product size
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATAT CCACT	101 bp
GLI1	AGGGAGGAAAGCAGACTGAC	CCAGTCATTTCCACACCACT	137 bp
SZIN1	GCCAGGAAGCGAAAACACAC AATC	GCCACTCTTGACCTCTCCATCTC	161 bp
SOX2	AGCTACAGCATGATGCAGGA	GGTCATGGAGTTGTACTGCA	126 bp

Table 2 Correlation of GLI1 mRNA expression with clinicopathological features of ESCC patients

Feature		GLI1 expression		*P* value
		Unchanged/Under (n)	Over (n)	
Sex	Male	20	3	0.035*
	Female	15	11	
Lymph node metastasis	No metastasis	21	6	
	Node metastasis	14	8	0.047*
Depth of tumor invasion	T1,2	7	4	0.511
	T3,4	28	10	
Surgical stage	Stage1,2	21	9	0.520
	Stage3,4	14	5	
Grade of differentiation	P. D	3	2	0.460
	M. D	25	8	
	W. D	7	4	
Location	Lower	20	2	0.002*
	Middle	15	10	
	Upper	0	2	

*Significant

Increased expression level of GLI1 in ESCC

Using real-time PCR, Overexpression of GLI1 was detected in ESCC. The mean (±SD) of gene expression in ESCC tissues was 0.99 (± 1.86). The lower and upper fold changes of gene expression were −4.80 and 6.43, respectively. GLI1 was significantly overexpressed in 14 out of 49 patients (28.6%, *p* value <0.05).

Correlation of GLI1 gene expression and clinicopathological features of ESCC patients

The correlation between GLI1 mRNA expression and different clinicopathological characteristics of ESCC patients was analyzed. The GLI1 overexpression was correlated with age ($p = 0.02$). According to the results, 88.2% of studied population were over 40 years old. GLI1 overexpression was associated with the increased range of age. There was also correlation between increased level of GLI1 expression and location of tumor ($p = 0.002$). 51% (25 out of 49) of ESCCs were resected from the middle part of esophagus which 40% (10 out of 25) of these tissues showed GLI1 overexpression. Furthermore, a significant correlation between GLI1 overexpression and lymph node metastasis was observed ($p < 0.05$). 22 of 49 patients (44.9%) had lymph node metastasis which GLI1 was overexpressed in 36% (8 out of 22) of these samples. Although GLI1 gene expression was not significantly correlated to the surgical stage of the disease, the majority of GLI1 overexpressed samples (9 of 14) were categorized into primary surgical stages (stages 1 and 2), while the others (5 of 14) showed advanced stages (surgical stages 3 and 4).

Correlation of GLI1 with SOX2 and SIZN1 in ESCC

The Pearson correlation and regression model indicated the significant correlation of GLI1 with SOX2 and SIZN1 in ESCC. The results showed significant correlation between GLI1 and SOX2 ($P = 0.011$, correlation coefficient: 0.360) and SIZN1 ($p = 0.010$, correlation coefficient: 0.363) in ESCC (Tables 3 and 4). It means that in tumor sample with GLI1 overexpression the level of SOX2

Table 3 Correlation between GLI1 and SOX2 mRNA expression in ESCC

Pearson correlation	SOX2		*P* value	
	Unchanged/Underexpression	Overexpression		
GLI1				
Unchanged/ Underexpression	27	8	35	
Overexpression	6	8	14	0.011*
Total	33	16	49	

*Significant correlation

Table 4 Correlation between GLI1 and SIZN1 mRNA expression in ESCC

Pearson correlation	SIZN1		*P* value	
	Unchanged/Underexpression	Overexpression		
GLI1				
Unchanged/ Underexpression	30	5	35	
Overexpression	8	6	14	0.010*
Total	38	11	49	

*Significant correlation

and SIZN1 gene expression were also increased (the data of SIZN1 expression in ESCC is unpublished). In 49 patients, 16.3% had overexpression of both GLI1 and SOX2. Concomitant overexpression of GLI1 and SIZN1 was detected in 12.3% of samples. In addition to Pearson correlation, regression model for GLI1 as dependent and SOX2 as well as SIZN1 as independent variables illustrated the significant correlation between GLI1 and the genes (Fig. 1).

Discussion

Stemness state signaling pathways play role in tumorigenesis. The crosstalk between these pathways is important to understand cancer system biology, leading to predict new biomarkers and effective therapeutic targets (Werner et al. 2014; Forghanifard et al. 2015). It has been shown that EMT and stemness state pathways are linked together in different aspects (Mani et al. 2008; Morel et al. 2008). It is also indicated that EMT is a complicated process which conducted with a variety of TFs (Forghanifard et al. 2012). Since expression of TFs is different in tumor cells, the interaction among such TFs may be a cause of tumor heterogeneity (Sun and Yu 2015). In the present study, gene expression of GLI1 was assessed in ESCC patients and its correlation with lymph node metastasis was detected. The increased GLI1 expression was correlated to the SOX2 and SIZN1 gene expression, which may introduce a new crosstalk between Hh and stemness state pathways.

A couple of studies have reported correlation of GLI1 expression with lymph node metastasis and EMT in ESCC (Min et al. 2013; Mori et al. 2007). S Min et al. demonstrated that GLI1 promotes the EMT via SNAIL expression and E-cadherin inhibition (Min et al. 2013). In line with these reports, current study was also showed the upregulation of GLI1 mRNA in ESCC in significant correlation with lymph node metastasis, emphasizing the link between GLI1 expression and downstream associated

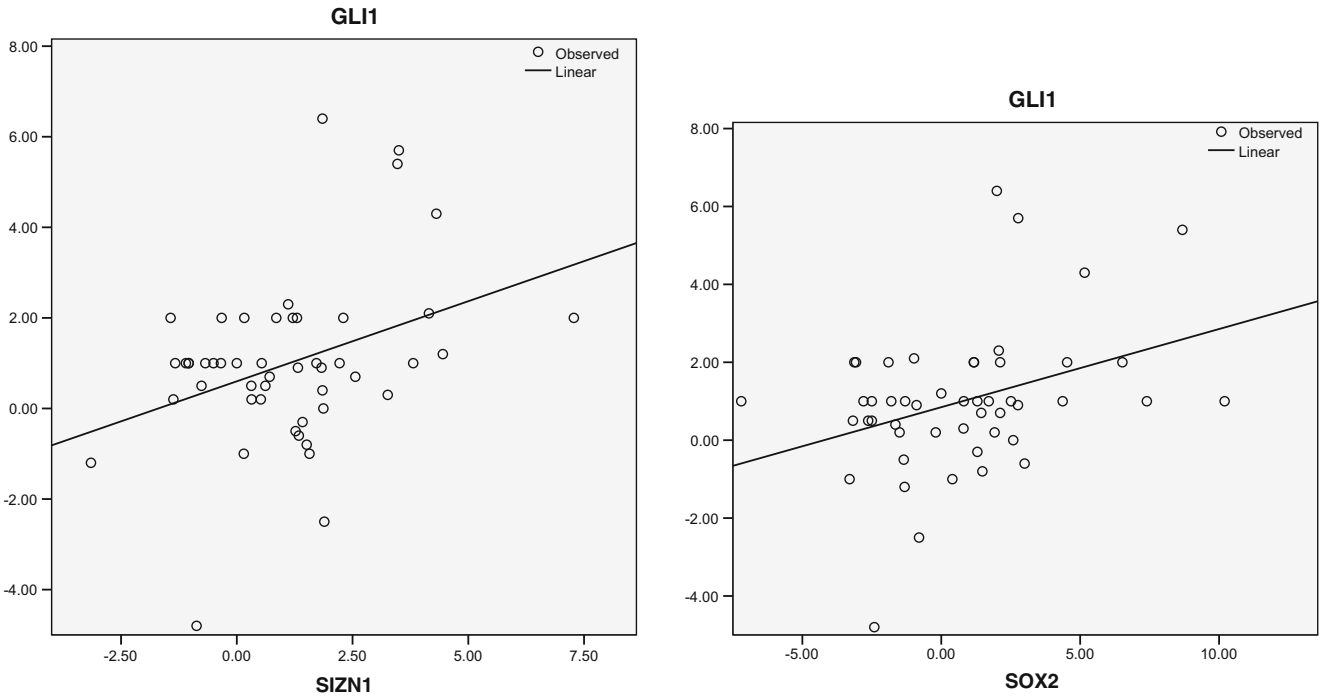

Fig. 1 Correlation between GLI1 and the genes (SOX2 and SIZN1) is depicted as regression plot. The samples with high level of GLI1 expression show elevated level of SOX2 and SIZN1 expression as well. The X and Y axis show fold change of gene expression

genes with EMT in ESCC. Our recent reports indicated that EMT and stem cell markers have main role in progress of ESCC (Forghanifard et al. 2012, 2016a, b). The results from gene expression analysis of different stem cell pathways in ESCC and their correlation with GLI1, statistically predicts correlation of GLI1 and critical TFs including SOX2 and SIZN1 genes.

It has been shown that SOX2 was correlated statistically with SALL4 and MEIS1, in significant association with lymph node metastasis of ESCC (Rad et al. 2016; Forghanifard et al. 2014b). In this study, the increased level of GLI1 was associated with overexpression of SOX2 in ESCC which may be resembled similar regulatory process in non-small cell lung cancer (NSCLC). It was reported that GLI1 regulates SOX2 expression through its promotor in NSCLC (Bora-Singhal et al. 2015). Varnat et al. also showed the Hh signaling regulates the embryonic stem-like signature components including NANOG, SOX2, OCT4 and KLF4 in colon cancer, which the level of these TFs increased after enhanced expression of GLI1 (Varnat et al. 2010). Furthermore, GLI1 regulates SOX2 through crosstalk of SHH-BMP signaling pathway in epithelial stem cell maintenance during molar development. It has been shown that BMP signaling cascade affects GLI1 to regulate the SOX2 expression and control epithelial stem cell fate (Li et al. 2015). In line with these reports, our study confirmed overexpression of both genes in poorly differentiated ESCC cases with advanced surgical stages (stages III and IV). It may suggest that GLI1 through SOX2 activation maintains the cells in poorly differentiated state (Fig. 2).

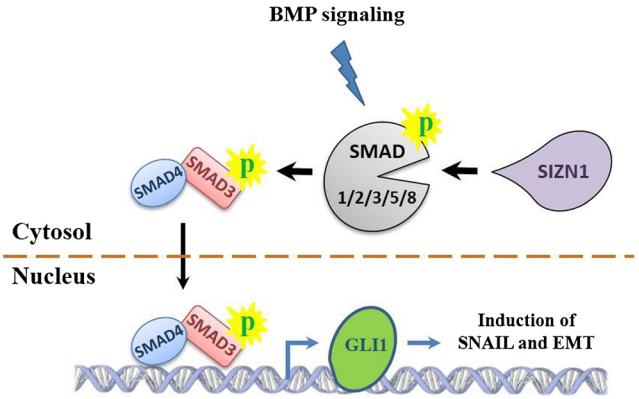

Fig. 3 SIZN1 may involve in EMT induction through GLI1 expression and BMP signaling. SIZN1 interacts with the receptor-regulated SMADs (SAMD1/2/3/5/8) to co-activate the signaling and recruit SMAD4. GLI1 expression is enhanced following SMAD4/SMAD3 binding to its regulatory region, and subsequently GLI1 induces the SNAIL expression

SIZN1, zinc finger CCHC-type containing 12 (ZCCHC12), encodes a downstream effector of BMP signaling and modulates this pathway by interacting with SMAD family members, especially SMAD1, and associates with the cAMP-responsive element-binding protein (CREB) (Cho et al. 2008). Although, there is rare literature reviews about the SIZN1 expression in malignancies, its overexpression was

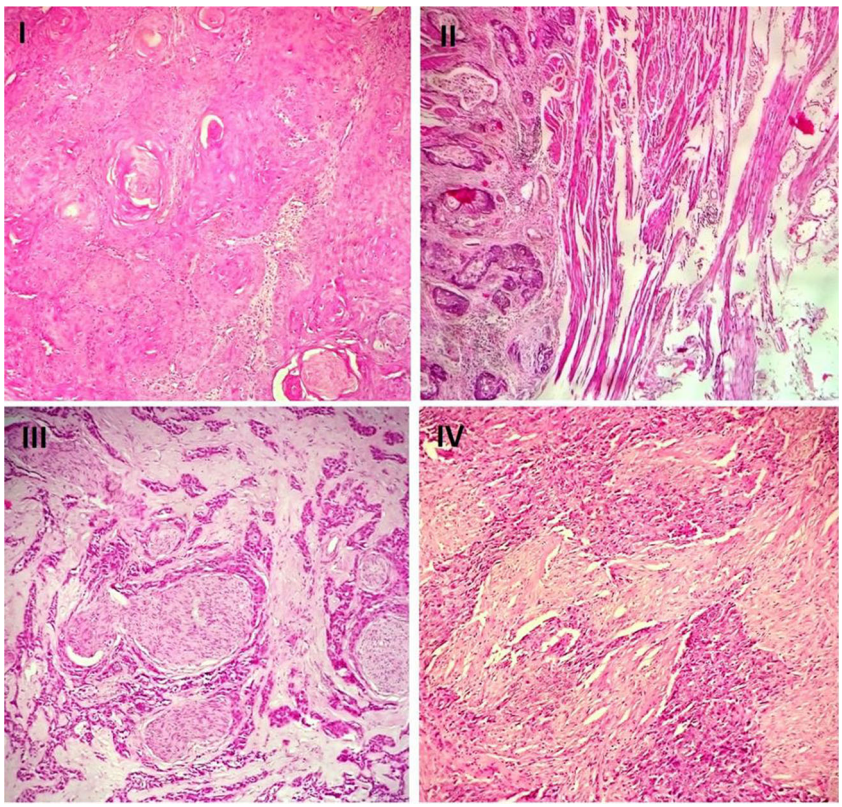

Fig. 2 Histological pictures of four ESCC cases with different stages. I. A well differentiated squamous cell carcinoma with keratin pearl formation and minimal nuclear atypia. A stage I tumor with GLI1 overexpression (H&E stain, ×200). II. The moderately differentiated tumor invades muscularis propria with stage II. GLI1 and SOX2 overexpression was detected in this sample (H&E stain, ×100). III. The poorly differentiated tumor with perineural invasion in ESCC. A stage III tumor with GLI1 and SOX2 overexpression (H&E stain, ×200). IV. The poorly differentiated squamous cell carcinoma demonstrates pleomorphic cells with high nucleo-cytoplasmic ratio and elevated expression of SOX2. The patient had distant metastasis (stage IV) (H&E stain, ×200)

studied in papillary thyroid carcinoma (PTC) which was higher compared to nodular goiter (Li et al. 2012). In present study overexpression of GLI1 was correlated to the high level of SIZN1 expression. The association between GLI1 and SIZN1 may propose crosstalk between BMP and Hh signaling pathways. BMP signaling pathway initiates by transforming growth factor-β (TGF-β) followed by phosphorylation of Smad1/2/3/5/8. TGF-β is an inducer of EMT in cancers which cooperates with other developmental pathways to promote EMT. It has been shown that Hh components through TGF-β give rise breast cancer metastasis to bone (Javelaud et al. 2011). Jingyu et al., reviewed the induction of EMT by different pathways including TGF-β, SHH, and WNT (Zhang et al. 2016). It is proposed that TGF-β phosphorylates one of the receptor-regulated SMADs including Smad1/2/3/5/8, followed by recruiting of the SAMD4 to upregulate SNAIL. On the other hand, SMAD3/SMAD4 dimer upregulates the GLI1 expression. Regarding interaction of SIZN1 with SMAD family, it can be suggested that correlation of GLI1 with SIZN1 may be happened through TGF-β /EMT process crosstalk (Fig. 3).

In conclusion, this study predicted the association of increased level of GLI1 with SOX2 and SIZN1, the main TFs in stemness state and BMP signaling pathways, respectively. The correlation of GLI1 gene expression with lymph node metastasis, as well as its association with SOX2 and SIZN1, may suggest involvement of such cell signaling crosstalk in EMT promotion, as one of the main features of aggressive ESCC.

Acknowledgements We kindly acknowledge our colleagues at Division of Human Genetics, Immunology Research Center, Avicenna Research Institute (Mashhad University of Medical Sciences) for their technical supports.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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